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REMARKS

Claims 1, 3, 7-10, 12, 16-18, 34, 35 and 39 are pending in the instant application. Claims 1, 3, 7-10, 12, 16-18, 34, 35 and 39 have been rejected. New claims 40 through 43 have been added. Claims 8 and 17 have been canceled in light of the addition of claims 40-43. Support for these amendments is provided in the specification at page 17, line 20 through page 26, line 6, Examples beginning at page 44 and claims 8 and 17, now canceled. No new matter has been added by these amendments. Reconsideration is respectfully requested in light of these amendments and the following remarks.

Rejection of Claims 1, 3, 7-10, 12, 16-18, 34, 35 and 39 under 35 U.S.C. § 112, first paragraph

The rejection of claims 1, 3, 7-10, 12, 16-18, 34, 35 and 39 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement has been maintained. In particular, the Examiner suggests that the claims encompass transgenes which have not been disclosed or described in the specification. Further, the Examiner suggests that the working examples provided do not seem to provide adequate written description for the genus of transgenes claimed.

Applicants respectfully traverse this rejection. MPEP § 2163 is clear; description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces. Instead, satisfactory disclosure of a representative number is dependent upon one of skill's recognition that applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. In the instant case, Applicants have demonstrated efficacy of cellular immunogens comprising 2 different proto-oncogenes. See Examples 1 and 2. In addition, Applicants have provided a detailed list beginning at page 17 of the specification of additional proto-oncogenes of the genus useful in the present invention. Further, Applicants are providing herewith a reference by Cefai et al. published subsequent to the filing date of the instant application describing similar experiments demonstrating efficacy of the HER2/*neu* proto-oncogene as a cellular immunogen. A courtesy copy of Cefai et al. Intl. J. of Cancer 1999 83:393-400 is provided herewith.

Thus, it is believed that claims drawn to use of a genus of proto-oncogenes are adequately supported by the written

description of the specification. Teachings at page 17-26 of the instant specification make clear that applicants were clearly in possession of a sufficient number of species supportive of a genus as claimed. Further, the instant specification and its teachings clearly place the public in possession of a multitude of species of proto-oncogenes supportive of a claim to the genus.

Thus, the instant specification and the pending claims meet the "essential goal" of the written description requirements of 35 U.S.C. § 112, first paragraph as set forth in MPEP § 2163.

Further, in an earnest effort to advance the prosecution of this case, Applicants have added new claims 40-41 drawn to the proto-oncogenes specifically disclosed and/or described in the specification at pages 17- 26. Claim 40-41 are clearly supported by the written description of the specification. Teachings at page 17-26 of the instant specification make clear that applicants were clearly in possession of the invention as set forth in claims 40-41. Further, the instant specification and its teachings clearly place the public in possession of the invention as claimed in claims 40-41.

Applicants have also added new claims 42 and 43 drawn to proto-oncogenes demonstrated through either working examples in the specification or through subsequent publications to function

in accordance with the claimed invention. Specifically, claims 42 and 43 are drawn to cellular immunogens for immunizing a host against the effects of the product of a target proto-oncogene and methods for preparing these cellular immunogens where the target proto-oncogene is selected from the group consisting of *c-erbB-2* (HER2/*neu*), *c-myc and c-src*. *myc* and *src* proto-oncogenes are demonstrated by the working examples provided at pages 44-54 of the instant specification to provide effective cellular immunogens in accordance with the claimed invention while the teachings of Cefai et al. confirm teachings of the specification concerning the utility of the proto-oncogene *c-erbB-2* (HER2/*neu*) as a cellular antigen as claimed. These new claims thus also meet the written description requirements of 35 U.S.C. § 112, first paragraph.

Withdrawal of this rejection under 35 U.S.C. § 112, first paragraph is respectfully requested in light of the above remarks.

Conclusion

Applicants believe that the foregoing comprises a full and complete response to the Office Action of record. Accordingly,

favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

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Int. J. Cancer: **83**, 393–400 (1999)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

TARGETING HER-2/neu FOR ACTIVE-SPECIFIC IMMUNOTHERAPY IN A MOUSE MODEL OF SPONTANEOUS BREAST CANCER

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The identification of tumor-associated antigens has led to increased interest in vaccination strategies to treat and/or prevent cancer. This study examined the feasibility of active-specific immunotherapy against the breast-tumor antigen HER-2/neu using a HER-2/neu transgenic (rNeu-TG) mouse model. rNeu-TG mice develop spontaneous breast tumors after pregnancy, indicating that they fail to mount an effective immune response against rNeu. Allogeneic fibroblasts expressing HER-2/neu were used as a cell-based vaccine. Vaccination induced a rNeu-specific anti-tumor immune response that prevented tumor formation of transplanted breast-tumor cells, and also protected mice from spontaneous tumor formation. Both T-cell-mediated and humoral immune responses were detectable in vaccinated mice. Vaccination also protected tumor-bearing mice from a challenge with cell suspensions isolated from spontaneous tumors, indicating that rNeu-TG mice are not tolerant to rNeu, even after spontaneous tumor formation. However, established spontaneous tumors themselves were never affected. This observation correlated with T-cell infiltrations in the injected but not in the established spontaneous tumor. Thus, allogeneic fibroblasts are efficient vaccine vectors to prime a specific immune response against an over-expressed tumor antigen. Moreover, our results suggest striking differences in the immunological requirements for the rejection of an established vs. a transplanted tumor. *Int. J. Cancer* 83:393–400, 1999.
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Breast cancer accounts for more than 25% of cancer in women in Western societies. Despite improved survival of high-risk patients after adjuvant therapy, the long-term prognosis remains poor. A number of high-risk prognostic factors have been identified, including tumor size, axillary lymph-node metastases, lack of estrogen/progesterone receptor and over-expression of the proto-oncogene HER-2/neu (Clark, 1996).

HER-2/neu (c-erbB-2) is a member of the EGF-receptor (EGFR) family, and was originally identified by its ability to transform NIH3T3 fibroblasts (Di Fiore *et al.*, 1987). Further analysis showed that this membrane protein has high constitutive protein-tyrosine-kinase activity. Over-expressed HER-2/neu can form heterodimers with other members of the EGFR family (Tzahar *et al.*, 1996) and increase their affinity for their respective ligands. This results in transphosphorylation and enhanced signaling that ultimately lead to malignant transformation of the cell (Pinkas *et al.*, 1996). Consistent with this, HER-2/neu transgenic (rNeu-TG) mice form spontaneous breast tumors, indicating that HER-2/neu over-expression alone is sufficient to promote tumor formation *in vivo* (Muller *et al.*, 1988).

Over-expression of non-mutated HER-2/neu is found in 25 to 30% of tumors in breast-cancer patients (Slamon *et al.*, 1987). Selective alterations of expression in a tumor are a prerequisite for a tumor-associated antigen (TAAg) to be a target of an immune-mediated rejection. Since the first characterization of MAGE-1 (van der Bruggen *et al.*, 1991), various TAAgs have been identified, which are selectively recognized by cytotoxic T lymphocytes (CTLs). However most defined TAAgs are not tumor-specific and may also be expressed on normal tissues from which tumors arise. Although HER-2/neu is constitutively expressed at low levels on different normal adult tissues (Press *et al.*, 1990), HER-2/neu-specific CTLs and antibodies were detected in some patients (Disis *et al.*, 1994). This indicates that immunological tolerance to HER-2/neu as a self-antigen can be overcome. Therefore, HER-2/neu is a clinically relevant TAAg and appears a candidate of choice for active specific immunotherapy (ASI).

Several documented mechanisms may contribute to the failure to mount an efficient immune response against an established tumor: (i) tumor cells may fail to mount a clinically efficient immune response and the tumor is ignored (Ohashi *et al.*, 1991; Speiser *et al.*, 1997); (ii) tumors may induce peripheral tolerance/anergy to the TAAg(s) (Staveley-O'Carroll *et al.*, 1998; Ye *et al.*, 1994); (iii) tumors may develop mechanisms to escape immune-mediated rejection (Pawelec *et al.*, 1997).

In this study, we investigated the feasibility of ASI against HER-2/neu in rNeu-TG mice in which the mouse-mammary-tumor virus LTR targets the over-expression of the activated rat neu (rNeu) to the breast. Spontaneous breast tumors develop after a first litter (Muller *et al.*, 1988). This is a unique situation, where a single TAAg is associated with tumor formation. This model allowed us to address the following issues: (i) can a vaccine induce a specific immune response against spontaneous tumors expressing HER-2/neu? (ii) can tumor-bearing mice still mount an efficient anti-tumor immune response after vaccination? (iii) do established tumors have distinctive properties that enable them to escape immune-mediated rejection?

We show that vaccination with allogeneic rNeu-expressing fibroblasts can induce a specific and effective immune response to rNeu and prevent tumor formation. Our results further suggest that $rNeu^+$ established breast tumors do not induce a systemic immune defect, but rather develop mechanisms to escape immune rejection.

MATERIAL AND METHODS

Mice

FVB/NHd mice and MMTV/r-Neu FVB mice transgenic for the rat neu protein (rNeu-TG) were purchased from Charles River, Sulzfeld, Germany. The rNeu-TG mice harbor the activated full-length rat neu (rNeu) gene, mutated in the transmembrane region. This gene expression is under the control of the mouse-mammary-tumor-virus-LTR (MMTV) promoter/enhancer (Muller *et al.*, 1988). Animal care was in accordance with institutional guidelines. Transgenic animals were identified by PCR. DNA extraction was performed from tail tissue. A 500-bp fragment of rNeu was amplified by PCR using 30 cycles, as follows: denaturation, 94°C/1 min; annealing, 55°C/1 min; elongation, 72°C/3 min. The following primers were used: 5′-GGAACCTTACTTCTGTG-

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GTGTGAC-3′ (5′ primer), and 5′-TAGCAGACACTCTATGCCT-GTGTG-3′ (3′ primer). Presence of the 500-bp fragment was analyzed on 1% agarose gels.

Cell lines and antibodies

BALB/c3T3 ($H2^d$) fibroblasts (5×10^6) were transfected by electroporation either with 1 μg of SV2-Neo-SP65 alone (BALBc/mock) or together with 10 μg of pBR322-rNeu plasmid (BALBc/neu) in which the activated full-length rNeu gene expression is under the control of the Moloney-virus-LTR promoter/enhancer. G418-resistent $rNeu^+$ clones were selected by indirect immunofluorescence. Cells were incubated at 4°C for 30 min with the c-neu-specific MAb Ab-4 (Oncogene Science, Tarzana, CA), then incubated with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) and analyzed for fluorescence on a FACScan (Becton Dickinson, Heidelberg, Germany). The $rNeu^+$ ($H\text{-}2^q$) breast-cancer cell line NF9006 derived from a rNeu-TG mouse and the $rNeu^-$ ($H\text{-}2^q$) breast-cancer cell line M/BB 659 derived from a c-myc-TG mouse have been described (Morrison and Leder 1994).

Vaccination protocols

For vaccination/challenge studies, mice were vaccinated i.p. with 5×10^6 live BALBc/neu or BALBc/mock fibroblasts (0.5 ml in PBS) and boosted 2 weeks later with the same vaccination regimen. Another 2 weeks later, mice were challenged in the back by s.c. injection of either 2×10^6 NF9006 cancer cells or 2×10^6 cells isolated from a syngeneic $rNeu^+$ spontaneous breast tumor. For the isolation of tumor cells, freshly collected spontaneous breast tumors from rNeu-TG mice were cut into small pieces and incubated for 30 min at 37°C with collagenase and DNase I (Sigma, Buchs, Switzerland). The cell suspension was assessed for viability and injected immediately. For prophylactic vaccinations, virgin rNeu-TG female mice were injected i.p. and boosted 2 weeks later with 5×10^6 BALBc/neu fibroblasts. Mice were then allowed to mate, to activate the rNeu TG expression, and were regularly boosted every 2 weeks or left unvaccinated.

Surgical techniques and tumor implantation

Surgical procedures were performed after anesthesia with ketamine hydrochloride/xylazine (Ketalar, Parke-Davis, Baar, Switzerland, 100 μg/g body weight; Xylapan, Chassot, Bern, Switzerland, 8 μg/g body weight) in aseptic conditions, a dissecting microscope (type 334790, Wild, Heerbrugg, Switzerland) being used. Mice (3–4 months old) were fitted with dorsal skinfold chambers. After 2 days, a small piece (diameter approx. 1 mm) from a freshly extracted spontaneous mammary tumor was implanted into the chamber. Tumor growth was monitored daily by an Axioplan intravital microscope (Zeiss, Oberkochen, Germany). Mice were killed at indicated time points, and tissues were collected for histological and immunohistochemical analysis.

Histology and immunohistochemistry

For histological analysis, de-paraffinized slides were stained with hematoxylin/eosin. For immunohistochemical studies, de-paraffinized slides were incubated for 1 hr with rabbit anti-HER/2-neu C-18 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-CD3 (Dako, Copenhagen, Denmark) Abs diluted in TCGA buffer (Tris buffer, 50 mM, pH 7.5, supplemented with 1% casein, 0.02% NaN_3 and 5% normal goat serum). Slides were washed and further incubated for 45 min with 1/200 dilution of biotinylated goat anti-rabbit Abs (Dako) in TCGA. Reactivities were detected using alkaline-phosphatase-conjugated avidin-biotin complex (Dako) and Newfuchsin substrate (Sigma) according to the manufacturer's instructions. Slides were counterstained with hematoxylin.

In vivo *CD4 and CD8 T-cell depletion*

Rat anti-CD4 (GK1.5) and rat anti-CD8 (YTS.169, a kind gift from Dr. H. Waldmann, Cambridge) MAbs were purified from cell-culture supernatants using a protein-G affinity column (Pharmacia, Dübendorf, Switzerland). Mice were injected i.p. with 250 μg GK1.5 and/or 250 μg YTS.169 at days 6, 5 and 4 before challenge with spontaneous tumor cells and every 3 to 4 days thereafter. This routinely led to complete depletion of CD4 and CD8 T-cell subsets (not shown).

FACS analysis for determination of rNeu-specific antibody levels

$RNeu^+$ NF9006 cells (0.5×10^6) were incubated with mouse sera (1:100 dilution) for 45 min at 4°C in PBS supplemented with 5% goat (PBSAG) or rat (PBSAR) normal serum. Cells were then washed and further incubated for 30 min with 10 μg/ml FITC-conjugated goat $F(ab')_2$ anti-mouse IgG(H + L) (Southern Biotechnology) in PBSAG, FITC-conjugated rat anti-mouse IgG_1 in PBSAR or goat anti-mouse IgG_{2a} in PBSAG (both Abs from PharMingen, Hamburg, Germany). Cells were analyzed for fluorescence on a FACScan (Becton Dickinson).

RESULTS

Generation of HER-2/neu transfected BALB/c fibroblasts

As shown in Figure 1, BALB/c fibroblasts transfected with rNeu (BALBc/neu) expressed significant amounts of cell-surface rNeu as monitored by immunofluorescence staining. No rNeu expression was detected on BALBc/mock fibroblasts transfected with the SV2-Neo-SP65 plasmid alone. Also shown is the rNeu staining of 2 syngeneic breast-cancer cell lines further used for challenge experiments. The $rNeu^+$ breast-cancer cell line NF9006 expressed high amounts of rNeu, while no rNeu expression was detected on the Neu- M/BB 659 cell line.

Induction of a rNeu-specific immune response in rNeu-TG mice

Virgin non-transgenic FVB and virgin rNeu-TG mice ($H\text{-}2^q$) were either not vaccinated, or vaccinated and boosted with allogeneic BALBc/mock or BALBc/neu fibroblasts ($H\text{-}2^d$). Two weeks later, mice were challenged with the syngeneic Neu^+ breast-cancer cell line NF9006 ($H\text{-}2^q$) and assessed for tumor formation at the challenge site. As shown in Table I, all unvaccinated or BALBc/mock-vaccinated mice developed tumors at the

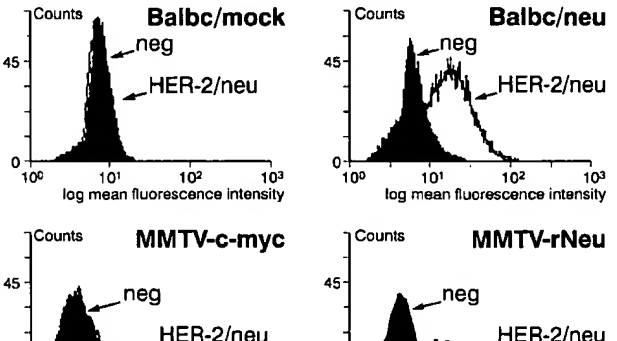

FIGURE 1 – FACS analysis of rNeu-transfected fibroblasts and breast-cancer cell lines. BALB/c fibroblasts transfected with a control neomycin vector (BALBc/mock) or with a vector expressing rNeu (BALBc/neu), $rNeu^+$ NF9006 (MMTV-rNeu) and $rNeu^-$ MM/B659 (MMTV-c-myc) breast-cancer cell lines were analyzed for rNeu expression by indirect immunostaining, using the rNeu-specific MAb Ab-4, followed by FITC-conjugated goat anti-mouse IgG (unshaded). Dark shaded area indicates control staining with FITC-conjugated goat anti-mouse IgG alone.

TABLE I – VACCINATION WITH BALBc/neu CELLS PREVENTS INJECTED TUMOR FORMATION

Vaccination $H2^d$	Challenge $H2^q$	Tumor incidence		
		FVB $H2^q$ virgin	rNeu-TG $H2^q$ virgin	rNeu-TG $H2^q$ after 1st pregnancy
No vaccination		10/10	10/10	6/6
BALBc/mock i.p.	$rNeu^+$	9/10	10/10	5/5
BALBc/neu i.p.		0/10	0/10	0/8

Female FVB ($H\text{-}2^q$) or rNeu-TG ($H\text{-}2^q$) mice, either virgin or 4 weeks after a first litter, were either not vaccinated or vaccinated/boosted i.p. with 5×10^6 BALBc/mock or BALBc/neu cells ($H\text{-}2^d$). 2 weeks after boost, each group was challenged s.c. with 2×10^6 syngeneic $rNeu^+$ NF9006 breast-tumor cells ($H\text{-}2^q$). Tumor formation was assessed at the challenge site. The results combine 2 independent experiments.

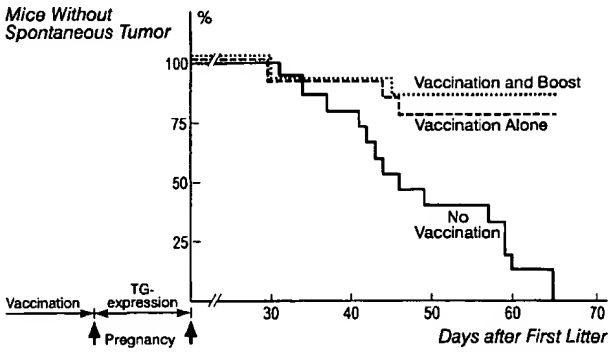

FIGURE 2 – Effect of prophylactic vaccination on spontaneous breast-cancer formation. Virgin rNeu-TG mice (8–10 weeks) were vaccinated and boosted with BALBc/neu cells, as described in Table I. Mice were allowed to mate to induce transgene expression. One group of mice was then left untreated (vaccination alone), while another group was further boosted with 2×10^6 BALBc/neu fibroblasts every 2 to 3 weeks (vaccination and boost). The time from first litter to development of palpable spontaneous breast cancer was assessed. These 2 groups (n = 15) were compared with a control group of unvaccinated (n = 15) and continually mated rNeu-TG mice (no vaccination).

challenge site. In contrast, none of the BALBc/neu-vaccinated mice developed tumors for an observation period of more than 2 months. BALBc/neu fibroblasts did not protect mice from challenge with the $rNeu^-$ syngeneic cell line M/BB 659 (data not shown).

The same vaccination experiments were carried out in rNeu-TG mice, starting at 4 weeks after a first litter. At this time point, no palpable tumor was detected, but histologic analysis showed that rNeu was expressed in the breast on rare microtumors scattered throughout the normal mammary gland (not shown). Mice were also protected from a challenge with NF9006 cells after vaccination with BALBc/neu but not with BALBc/mock cells. These results indicate that allogeneic BALBc/neu fibroblasts induce a rNeu-specific anti-tumor response in rNeu-TG mice after transgene expression.

Prophylactic vaccination with BALBc/neu fibroblasts significantly inhibits spontaneous breast-cancer formation

We further investigated whether vaccination with BALBc/neu cells could prevent the development of spontaneous breast tumors. Virgin female rNeu-TG mice were vaccinated and boosted with BALBc/neu cells and then allowed to mate continually to upregulate rNeu-transgene expression. Mice were then kept without further boosting, or were boosted every 2 to 3 weeks with BALBc/neu cells and monitored for tumor formation. As shown in Figure 2, all unvaccinated and continually mated rNeu-TG mice developed spontaneous breast tumors within 65 days after their first litter (range, 31–65 days). At this time point, 80% (12 out of 15) of vaccinated mice and 87% (13 out of 15) of vaccinated/boosted rNeu-TG mice were still tumor-free. Mice receiving vaccination with or without boost had no difference in their time to tumor development ($p = 0.65$). Thus, prophylactic vaccination with BALBc/neu cells significantly prevented the development of spontaneous breast tumors, in comparison with unvaccinated controls ($p < 0.00001$ for both vaccinated groups by Fisher's exact test).

Involvement of T cells in the rejection of $rNeu^+$ tumor cells

To get further insight in the mechanisms responsible for tumor rejection after vaccination, small pieces of spontaneous tumors isolated from a rNeu-TG mouse were implanted into dorsal-skinfold chambers. This system enables continuous observation of the implant using intravital microscopy. Consistent with the rejection of injected tumor-cell suspensions (see Table I), the small pieces of (undissociated) tumors were also rejected in 5 out of 6 BALBc/neu-vaccinated mice, while angiogenesis and tumor growth were observed in all 6 unvaccinated mice (not shown). At day 4 after implantation, implanted tumors were excised *in toto*, including the host bed (striated skin muscle, subcutaneous tissue and epidermis) and analyzed histologically. In unvaccinated mice, the border of the tumor implant on top of the striated muscle was well demarcated from the neighboring normal tissue (Fig. 3*a*). Tumor cells appeared uniform, with a large, slightly granular cytoplasm, and some nuclei showed mitosis (Fig. 3*c*). No inflammatory infiltrates were detected. In vaccinated mice, however, the tumor border was not well delineated from the underlying striated muscle and a large amount of necrosis was found, predominantly in the center of the tumor (Fig. 3*b*). The surviving tumor cells appeared in contiguous groups separated by a significant infiltrate of small lymphoid cells also infiltrating the underlying skin muscle (Fig. 3*d*). Immunohistochemical staining showed that this infiltrate consisted of $CD3^+$ cells (Fig. 3*f*). In contrast, no CD3 staining was detected in the tumor implant of unvaccinated mice (Fig. 3*e*). The immunohistochemical staining of rNeu was uniform throughout the tumor in unvaccinated mice (Fig. 3*g*). In vaccinated mice, however, the rNeu staining was intense in cells with a large cytoplasm and was faint in tumor cells undergoing necrosis or in the vicinity of the $CD3^+$ cells (Fig. 3*h*).

Systemic depletion of $CD4^+$ and $CD8^+$ T cells by MAbs was performed. Mice were vaccinated and boosted with BALBc/neu cells. T-cell depletion was started one week later, at day 6 before challenge, and continued for the entire observation period thereafter. As shown in Figure 4, a challenge with cells freshly isolated from a syngeneic spontaneous breast tumor was rejected in all immunized mice (>60 days). In contrast, 6 of 10 CD8-depleted mice and 2 of 9 CD4-depleted mice developed tumors at the injection site. Moreover, tumors formed in 8 of 9 $CD4^+$- and $CD8^+$-depleted mice, and the rate of tumor growth was comparable with that of unvaccinated animals. This indicates that vaccination with BALBc/neu cells triggers a T-cell-mediated immune response and suggests that mainly $CD8^+$ and, to a lesser extent, $CD4^+$ T cells are involved in tumor rejection.

Induction of a humoral response to HER-2/neu after vaccination with BALBc/neu cells

We sought to determine whether vaccination with BALBc/neu fibroblasts also induced a rNeu-specific humoral immune response. rNeu-TG mice were vaccinated and boosted with BALBc/mock or BALBc/neu cells, and sera were collected at days 49 to 56 after the first vaccination. The presence of anti-rNeu antibodies was assessed by flow-cytometry analysis of NF9006 cells incubated with mouse sera, followed by FITC-conjugated goat anti-mouse IgG. As shown in Figure 5*a*, mice injected with BALBc/neu cells showed a high level of antibody binding (mean, 78.6; SEM, 11.9). No rNeu-specific antibodies were detected in sera from unvaccinated mice (mean, 11.3; SEM, 5.2). A slight increase of antibody binding

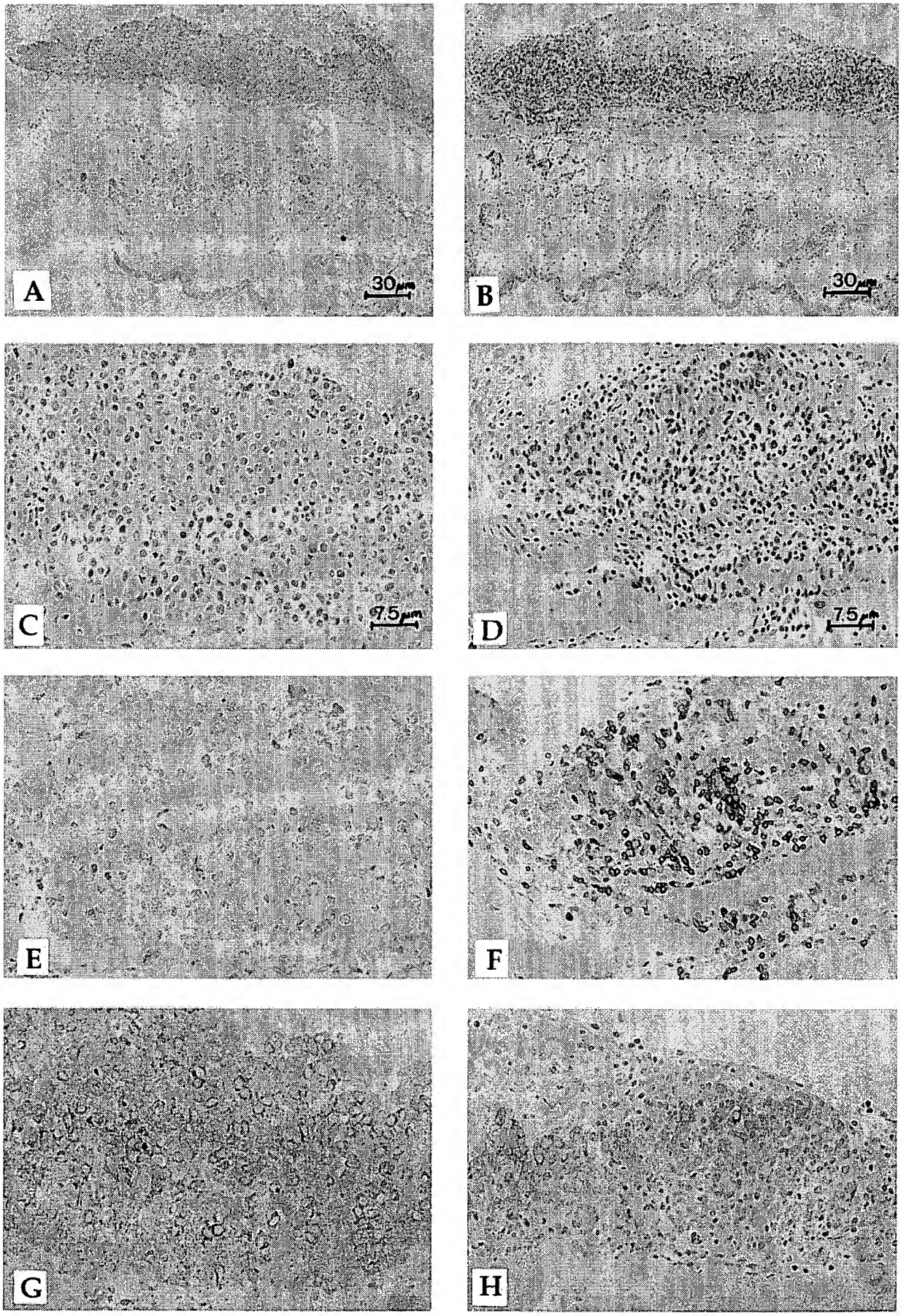

FIGURE 3

over baseline was noticed in BALBc/mock-vaccinated mice (mean 35.1; SEM 1.9). The same sera were tested for rNeu-specific IgG1 and IgG2a (Fig. 5*b*). Mice primed with BALBc/mock cells produced no rNeu-specific IgG1 or IgG2a. However, vaccination with BALBc/neu cells triggered a similar increase of both IgG1 and IG2a ($p < 0.0025$ for both groups by the Mann-Whitney rank test).

Established spontaneous breast tumors do not affect the systemic immune response against rNeu

To determine whether the presence of established tumors affects the ability to mount an immune response, rNeu-TG mice showing no more than one established spontaneous tumor (<5 mm) were vaccinated and boosted, or left unvaccinated, and challenged 2 weeks later with rNeu$^+$ cells freshly isolated from a spontaneous breast tumor. During the observation period of 45 days after challenge, only 1 of 8 vaccinated/boosted mice formed a tumor at the injection site on day 27 (Table II). In contrast, all 9 unvaccinated mice formed tumors at the injection site within 30 days (median time, 21 days, $p = 0.0034$), showing the same median time to tumor formation as unvaccinated tumor-free mice. This demonstrated that tumor-bearing mice were capable of mounting an effective anti-tumor response after vaccination. However, the pattern of spontaneous tumor growth was not different from that in unvaccinated mice, and all animals had to be killed due to progressive spontaneous tumors.

The presence of CD3$^+$ T cells in the transplanted and in the established spontaneous tumors was investigated. From 2 to 4 days after challenge, injected and established spontaneous tumors from the same vaccinated mice were collected and analyzed by standard hematoxylin/eosin and immunohistochemical staining for CD3. At the injection site, tumor cells appeared as scattered clusters of necrotic cells without apparent tissue-like organization (Fig. 6*a*). A significant infiltrate at the border of the tumor and in the surrounding connective tissue consisted of some monocytes and granulocytes and numerous small lymphocytes. Strong CD3$^+$ staining was observed, especially in close vicinity to the tumor cells (Fig. 6*b*). In comparison, granulocytes and monocytes, but only few lymphoid cells, were observed in unvaccinated tumor-bearing mice (not shown). In contrast, no significant infiltrate of lymphoid cells was observed in the established spontaneous tumors and the surrounding tissues of vaccinated mice (Fig. 6*c*). This was further confirmed by the absence of CD3 staining (Fig. 6*d*). These observations demonstrate the crucial role of T-cell infiltrates in tumor rejection, and reveal striking differences in the interaction of injected *vs.* established tumors with the host's immune system.

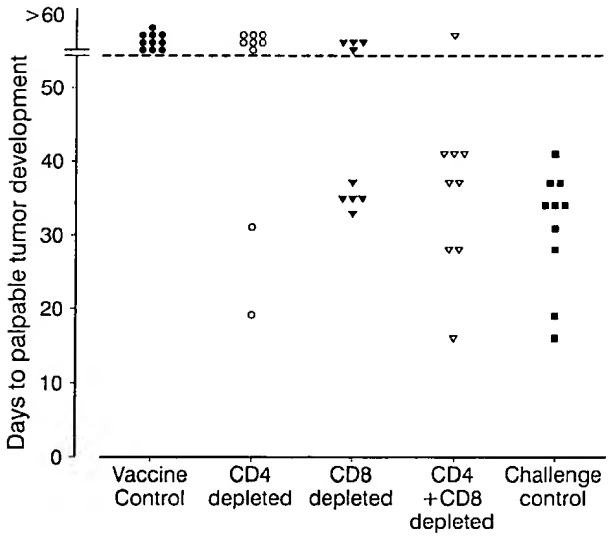

FIGURE 4 – Inhibition of tumor rejection after *in vivo* T-cell depletion. Tumor-free virgin rNeu-TG mice were vaccinated and boosted i.p. with BALBc/neu cells. Then, 1 week after the boost, mice were treated with PBS, anti-CD4 MAb (GK 1.5), anti-CD8 MAb (YTS 169.4) or anti-CD4 MAb and anti-CD8 MAb. Mice were then challenged s.c. in the back with 2×10^6 tumor cells from a spontaneous rNeu-TG breast tumor. Time to tumor formation at the challenge site was compared with control unvaccinated and vaccinated mice. Animals still tumor-free after 60 days are shown above the dashed bar.

FIGURE 3 – T-lymphocyte infiltration in tumor implants from rNeu-TG mice after vaccination with BALBc/neu. Virgin rNeu-TG mice were left untreated or vaccinated/boosted with BALBc/neu cells. A small piece (approx. 1 mm) of a syngeneic spontaneous breast tumor was implanted into dorsal skinfold chambers and collected at day 4 after implantation. *(a–d)* Paraffin sections of tumor implants from control *(a,c)* or BALBc/neu-vaccinated *(b,d)* mice were stained with hematoxylin/eosin. Note replacement of tumor cells by small lymphocytes along skin muscle and necrotic tissue in center of tumor *(b,d)*. *(e–h)* Immunohistochemical staining for CD3 and HER-2/neu. Sections from unvaccinated *(e)* and BALBc/neu-vaccinated *(f)* mice were stained for CD3 and counterstained with hematoxylin. The same tumor implants from unvaccinated *(g)* and vaccinated *(h)* mice were stained for HER-2/neu. Note intense staining in tumor cells with a large cytoplasm in unvaccinated mice *(g)*.

DISCUSSION

We show in this study that vaccination of HER-2/neu transgenic mice with allogeneic fibroblasts expressing rNeu (BALBc/neu) induces an anti-tumor immune response. Since HER-2/neu is expressed on various normal tissues (Press *et al.*, 1990), our first concern was that tolerance or auto-immunity might impair immunotherapy. BALBc/neu fibroblasts (H-2^d) injected into rNeu-TG mice (H-2^q) are likely to be degraded and to release rNeu, which can be endocytosed by bone-marrow-derived APCs of the host. rNeu can therefore access the MHC-class-I and -class-II processing pathways and be presented to T cells in spleen and lymph nodes. This "cross-priming" has been described for various exogenous antigens, in particular some TAAgs (Huang *et al.*, 1994; Toes *et al.*, 1996). This vaccination proved to be highly efficient, preventing the development of injected tumor cells in rNeu-TG mice, also specific, since it afforded no protection against a syngeneic rNeu$^-$ tumor cell line. Protection was mediated by T cells, since significant infiltration of T cells impressed in the implanted tumor, and T-cell-depleted mice were poorly protected after vaccination. Vaccination also induced a strong rNeu-specific humoral immune response. This indicates that rNeu-specific B and T cells pre-exist in the repertoire of FVB mice as functionally silent cells and that rNeu-TG mice are not tolerant to rNeu. Therefore, the efficacy of our vaccine may be due to its capacity to activate the rNeu-specific humoral and cellular arms of the immune system.

Prophylactic vaccination with BALBc/neu cells also protected more than 85% of rNeu-TG mice from developing spontaneous breast tumors at a time point when all unvaccinated animals had palpable spontaneous tumors. Efficient rejection of spontaneous tumors has been shown in other mouse models. However, most studies targeted highly immunogenic viral proteins artificially expressed in various organs (Ohashi *et al.*, 1991; Oldstone *et al.*, 1991; Speiser *et al.*, 1997), while over-expressed HER-2/neu is a cellular oncogene that naturally occurs in the breast. Furthermore, we noted that vaccination/boosted mice were capable of feeding their litters, and no morphological alterations or T-cell infiltrations were found in normal breasts or other organs (not shown). This suggests that the immune response was specifically directed towards over-expressed HER-2/neu on tumor cells.

Developing tumors or transgene expression correlated in other models with the failure of vaccination protocols, suggesting the induction of anergy (Staveley-O'Carroll *et al.*, 1998; Ye *et al.*,

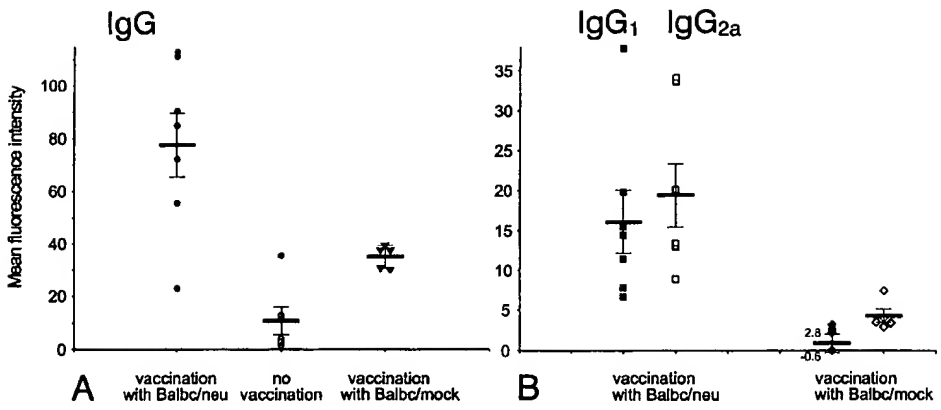

FIGURE 5 – Presence of serum IgG and isotypes against HER-2/neu in vaccinated rNeu-TG mice. RNeu-TG mice were vaccinated and boosted with BALBc/mock or BALBc/neu cells, as indicated, and sera were collected at days 49 to 56 after the first vaccination. NF9006 cells were incubated with indicated mouse sera (1:100 dilution), followed by FITC-conjugated MAbs specific for IgG (H + L), IgG1 or IgG2a, and analyzed for fluorescence by FACScan. *(a)* Mean fluorescence intensity for whole IgGs. *(b)* mean fluorescence intensity for IgG1 (closed symbols) and IgG2a (open symbols) in BALBc/mock- and BALBc/neu-vaccinated mice. Background fluorescence with FITC-conjugated anti-mouse MAbs alone was subtracted. Statistical analysis was by Mann-Whitney rank test.

TABLE II – CHALLENGE DEVELOPMENT IN TUMOR BEARING MICE

Tumor-bearing mice				
No vaccination		With vaccination		
Mouse number	Tumor at injection site (day)	Mouse number	Tumor at injection site	Observation time (days)
166	18	110	no tumor	28
167	14	164	no tumor	35
177	19	195	no tumor	45
190	28	220	no tumor	45
222	27	223	day 27	
269	27	224	no tumor	45
270	30	288	no tumor	40
272	17	289	no tumor	40
273	17			

RNeu-TG mice with palpable spontaneous breast tumors were either not vaccinated (no vaccination) or vaccinated and boosted i.p. with 5×10^6 BALBc/neu (with vaccination) as described in Table I; 2 weeks after booster injection, all mice were challenged s.c. in the back with 2×10^6 syngeneic rNeu$^+$ tumor cells isolated from a spontaneous breast tumor. Time to tumor formation (days) at the injection site is shown. The median time to tumor formation in the non-vaccinated group was 21 days (range, 14–30 days). Vaccinated mice were observed for 45 days and killed owing to spontaneous tumor progression.

1994). Antigen presentation in the absence of a co-stimulatory signal on tumor cells may lead to antigen-specific T-cell death or anergy (Guinan *et al.*, 1994). We showed that rNeu-TG mice over-expressing the transgene after a first litter, as well as mice with established spontaneous tumors, were as efficiently protected against a challenge with rNeu$^+$ cells as tumor-free mice after vaccination. This indicated that tumor-bearing mice had no systemic immune defect. Although we cannot entirely exclude the possibility that spontaneous tumors could induce partial anergy, this did not appear to be a dominant mechanism, or could be overcome by our vaccination protocol.

Although tumor-bearing animals could reject injected tumors after vaccination, established spontaneous tumors themselves were never affected, raising the possibility that established tumors had "escaped" the immune system. One possible explanation is that more effector cells are required for efficient regression of established tumors than can be triggered by our vaccination protocol. In transgenic mice expressing model proteins as self-antigen, efficient vaccination strategies have been shown to overcome tolerance and to induce autodestruction of the tissue expressing these antigens (Ohashi *et al.*, 1991; Oldstone *et al.*, 1991). However, we observed no protective effect when vaccination was started in rNeu-TG mice 4 weeks after their first litter. At this time point, the tumor burden was small (few microscopic tumors in the breast), but all vaccinated mice developed spontaneous breast tumors thereafter (not shown). Another explanation is that established tumors are directly responsible for the inefficacy of the immune response. Decreased MHC-class-I expression (Lollini *et al.*, 1998) or up-regulation of FasL (Walker *et al.*, 1998) have been associated with tumor escape in various tumor models. However, we showed that small undissociated pieces, as well as cell suspensions freshly derived from spontaneous tumors and transplanted into mice, were efficiently rejected and were unlikely to have altered expression of MHC-class-I or FasL molecules. Finally, the established tumor may have created a specific micro-environment that locally down-regulates the immune response. Consistent with this, significant CD3$^+$ infiltrates were observed in the same mouse at the challenge site of injected tumor cells (which were rejected) but not in the established spontaneous tumors after vaccination. It is likely that s.c. injection of tumor cells creates an inflammatory milieu that can up-regulate molecules such as members of the integrin and L-selectin family and favor the transendothelium migration of T lymphocytes (Springer, 1995). In contrast, the vascular endothelium in established spontaneous tumors may fail to express adhesion and integrin molecules that have been shown to target vaccination-activated T cells to the tumor (Arap *et al.*, 1998). However, we found that the rejection of injected tumors and the presence of T cells was strictly dependent on vaccination. Moreover, the failure to protect rNeu-TG mice with spontaneous microtumors in the breast from tumor progression indicates that tumor escape mechanisms may develop in early stages, with no relation to tumor burden.

Taken together, our results show that allogeneic fibroblasts, designed to obviate the need for HLA-restricted vaccines, efficiently prime a specific cellular and humoral immune response to over-expressed TAAg. They further suggest that the ability of rNeu$^+$ established breast tumors to avoid immune surveillance is related to mechanisms probably mediated by the tumor microenvironment. Injected tumors derived from these very spontaneous tumors were rejected, implying that challenge with tumor cell lines provide poor pre-clinical models for studying the mechanisms of immune-mediated rejection of spontaneous tumors.

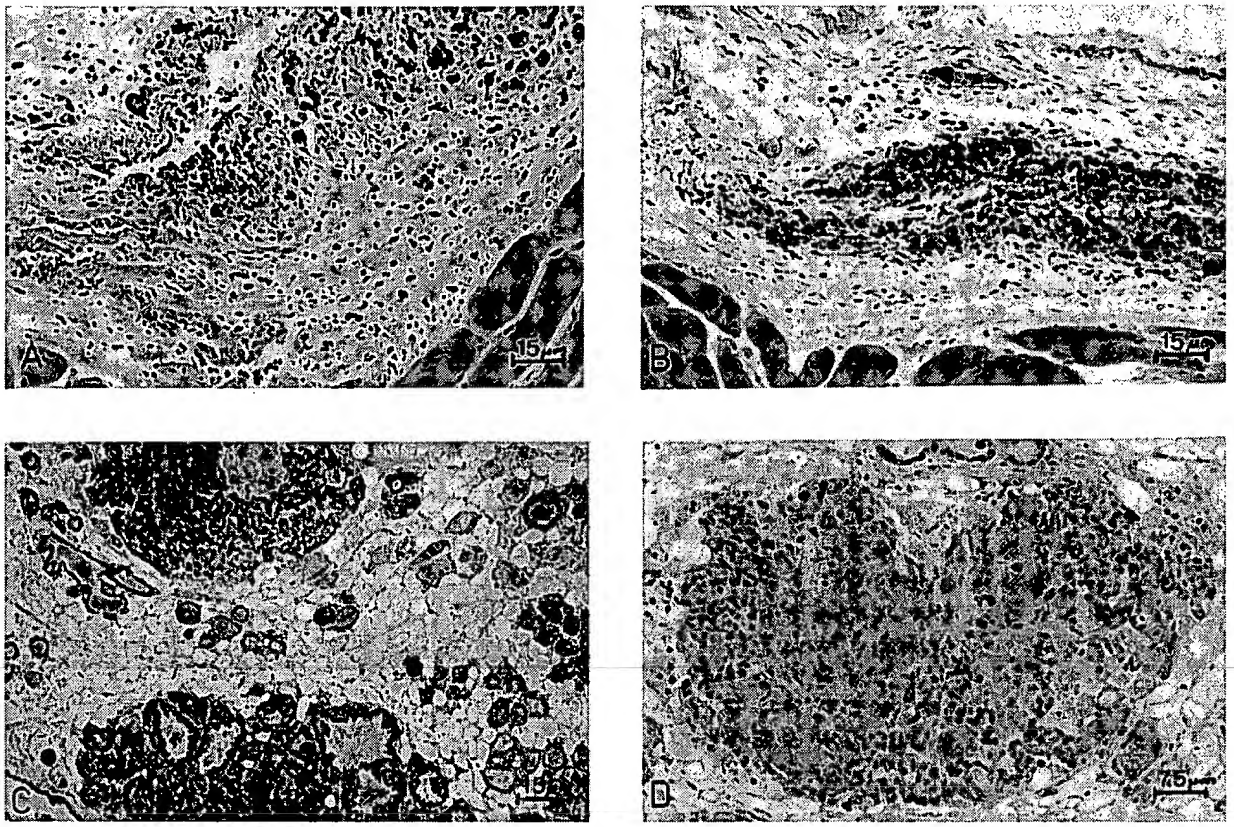

FIGURE 6 – Histological and immunohistochemical characterization of injected and established spontaneous breast tumors after vaccination. Tumor-bearing rNeu-TG mice were vaccinated/boosted and challenged s.c. with 2×10^6 tumor cells isolated from a spontaneous Neu^+ breast tumor; 2 days after challenge, established and injected tumors were collected. *(a)* Paraffin sections from injection sites were stained with hematoxylin/eosin. Note the infiltration of small lymphocytes in the center of the tumor. *(b)* Immunohistochemical staining for CD3 at the injection site shows cells with a characteristic peripheral red staining pattern, consistent with the cell-surface localization of CD3. *(c)* Standard hematoxylin/eosin staining of paraffin sections from an established spontaneous tumor in the same rNeu-TG mouse after vaccination. *(d)* Immunohistochemical staining, as in *(b)*, of an established spontaneous tumor for CD3.

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REFERENCES

ARAP, W., PASQUALINI, R. and RUOSLAHTI, E., Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science,* **279,** 377–380 (1998).

CLARK, G.M., Prognostic and predictive factors, 3rd ed., pp. 461–485, Lippincott-Raven, Philadelphia (1996).

DI FIORE, P.P., PIERCE, J.H., KRAUS, M.H., SEGATTO, O., KING, C.R. and AAARONSON, S.A., erbB-2 is a potent oncogene when overexpressed in NIH3T3 cells. *Science,* **237,** 178–182 (1987).

DISIS, M.L., CALENOFF, E., MCLAUGHLIN, G., MURPHY, A.E., CHEN, W., GRONER, B., JESCHKE, M., LYDON, N., MCGLYNN, E., LIVINGSTON, R.B., MOE, R. and CHEEVER, M.A., Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res.,* **54,** 16–20 (1994).

GUINAN, E., GRIBBEN, J., BOUSSIOTIS, V.A., FREEMAN, G.J. and NADLER, L.M., Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. *Blood,* **84,** 3261–3282 (1994).

HUANG, A.Y.C., GOLUMBEK, P., AHMADZADEH, M., JAFFEE, E., PARDOLL, D. and LEVITSKY, H., Role of bone-marrow-derived cells in presenting MHC-class-I-restricted tumor antigens. *Science,* **264,** 961–964 (1994).

LOLLINI, P.L., NICOLETTI, G., LANDUZZI, L., DE GIOVANNI, C., ROSSI, I., DI CARLO, E., MUSIANI, P., MULLER, W.J. and NANNI, P., Down-regulation of major-histocompatibility-complex-class-I expression in mammary carcinoma of HER-2/neu transgenic mice. *Int. J. Cancer,* **77,** 937–941 (1998).

MORRISON, B.W. and LEDER, P., *neu* and *ras* initiate murine mammary tumors that share genetic markers generally absent in c-*myc*- and *int*-2-initiated tumors. *Oncogene,* **9,** 3417–3426 (1994).

MULLER, W.J., SINN, E., PATTENGALE, P.K., WALLACE, R. and LEDER, P., Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. *Cell,* **54,** 105–115 (1988).

OHASHI, P., OEHEN, S., BUERKI, K., PIRCHER, H., OHASHI, C., ODERMATT, B., MALISSEN, B., ZINKERNAGEL, R. and HENGARTNER, H., Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell,* **65,** 305–317 (1991).

OLDSTONE, M.B.A., NERENBERG, M., SOUTHERN, P., PRICE, J. and LEWICKI, H., Virus infection triggers insulin-dependent diabetse mellitus in a transgenic model: role of anti-self(virus) immune response. *Cell,* **65,** 319–331 (1991).

PAWELEC, G., ZEUTHEN, J. and KIESSLING, R., Escape from host anti-tumor immunity. *Crit. Rev. Oncol.,* **8,** 111–114 (1997).

PINKAS, R., SOUSSAN, L., WATERMAN, H., LEVKOWITZ, G., ALROY, I., KLAPPER, L., LAVI, S., SEGER, R., RATZKIN, B., SELA, M. and YARDEN, Y., Diversification of the Neu differentiation factor and epidermal-growth-factor signaling by combinatorial receptor interactions. *EMBO J.*, **15**, 2452–2467 (1996).

PRESS, M.F., CORDON-CARDO, C. and SLAMON, D.J., Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissue. *Oncogene*, **5**, 953–962 (1990).

SLAMON, D.J., CLARK, G.M., WONG, S.G., LEVIN, W.J., ULLRICH, A. and MCGUIRE, W.L., Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*, **235**, 177–181 (1987).

SPEISER, D.E., MIRANDA, R., ZAKARIAN, A., BACHMANN, M.F., MCKALL-FAIENZA, K., ODERMATT, B., HANAHAN, D., ZINKERNAGEL, R.M. and OHASHI, P.S., Self antigens expressed by solid tumors do not efficiently stimulate naive or activated T cells: implications for immunotherapy. *J. exp. Med.*, **186**, 645–653 (1997).

SPRINGER, T.A., Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Ann. Rev. Physiol.*, **57**, 827–872 (1995).

STAVELEY-O'CARROLL, K., SOTOMAYOR, E., MONTGOMERY, J., BORRELLO, I., HWANG, L., FEIN, S., PARDOLL, D. and LEVITSKY, H., Induction of antigen-specific T-cell anergy: an early event in the course of tumor progression. *Proc. nat. Acad. Sci. (Wash.)*, **95**, 1178–1183 (1998).

TOES, R.E., BLOM, R.J., VAN DER VOORT, E., OFFRINGA, R., MELIEF, C.J. and KAST, W.M., Protective anti-tumor immunity induced by immunization with the completely allogeneic tumor cells. *Cancer Res.*, **56**, 3782–3787 (1996).

TZAHAR, E., WATERMAN, H., CHEN, X., LEVKOWITZ, G., KARUNAGARAN, D., LAVI, S., RATZKIN, B.J. and YARDEN, Y., A hierarchical network of inter-receptor interactions determines signal transduction by NDF/neuregulin and EGF. *Mol. cell. Biol.*, **16**, 5276–5287 (1996).

VAN DER BRUGGEN, P., TRAVERSARI, C., CHOMEZ, P., LURQUIN, C., DE PLAEN, E., VAN DEN EYNDE, B., KNUTH, A. and BOON, T., A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*, **254**, 1643–1647 (1991).

WALKER, P.R., SAAS, P. and DIETRICH, P.Y., Tumor expression of Fas ligand (CD95L) and its consequences. *Curr. Opin. Immunol.*, **10**, 564–572 (1998).

YE, X., MCCARRICK, J., JEWETT, L. and KNOWLES, B.B., Timely immunization subverts the development of peripheral non-responsiveness and suppresses tumor development in simian-virus-40-tumor-antigen-transgenic mice. *Proc. nat. Acad. Sci. (Wash.)*, **91**, 3916–3920 (1994).